Title:

Differential proteolytic activity in *Anisakis simplex s.s.* and *Anisakis pegreffii*, two sibling species from the complex *Anisakis simplex s.l.*, major etiological agents of anisakiasis.

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Abstract

Proteolytic activity was studied in two sibling species of *Anisakis* (Nematoda: Anisakidae), *A. simplex s.s.* and *A. pegreffii*, throughout their in vitro development from third larval stage (L3) from the host fish (L3-0h) to fourth larval stage (L4) obtained in culture. Proteases have a significant role in the lifecycle of the parasite and in the pathogen-host relationship. Proteolytic activity peaks were detected at pH 6.0 and 8.5. Protease activity was detected in all the developmental stages of the two species studied at both pH values. These pH values were used for assaying with specific inhibitors which permitted the determination of metalloprotease activity, and, to a lesser extent, that of serine and cysteine protease. Aspartic protease activity was only detected at pH 6.0. At this pH, L4 larvae showed higher proteolytic activity than L3 larvae in both species ($p<0.001$), the majority of activity being due to metalloproteases and aspartic proteases, which could be related to nutrition, especially the latter, as occurs in invertebrates. At pH 8.5, proteolytic activity was higher in *A. simplex s.s.* than in *A. pegreffii* ($p<0.01$). At this pH, the majority of activity was due to metalloproteases in all developmental phases of both species, although in L3-0h, the activity of these proteases was significantly higher ($p<0.03$) in *A. simplex s.s.* than in *A. pegreffii*. This could be related to the greater invasive capacity of the former. Serine proteases have frequently been implicated in the invasive capacity and pathogenicity of some parasites.This may be related to the significantly higher activity ($p\leq0.05$) of serine protease in all the larval stages studied of *A. simplex* at pH 6.0. In summary, there

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are interspecific differences in proteases that have been related to pathogenesis in nematodes. These differences could thus be contributing to the previously reported differences in pathogenicity between these two *Anisakis* species.

Key words: nematodes; parasites; anisakiasis; sibling species of *Anisakis simplex s.l.*; peptidases

1.- Introduction

Anisakidosis is an illness caused by the third larval stages (L3) of anisakid nematodes. Although often undiagnosed, it is common in countries where fish or squid are typically consumed raw or only lightly cooked, such as Japan, where there are thought to be between 2000 and 3000 cases annually (Umehara et al., 2007). There are also significant numbers of cases in other countries with high consumption of fish, including Spain, Italy and South Korea (González Quijada et al., 2005; Im et al., 1995; Lim et al., 2015; Pampiglione et al., 2002; Repiso Ortega et al., 2003), as well as in many other countries throughout the world.

More than 97% of cases of anisakidosis are caused by the larvae of the *Anisakis simplex s.l.* complex (Rello Yubero et al., 2004), for which reason the infection is also known as anisakiasis. This complex comprises 3 species whose L3 are morphologically indistinguishable but can be differentiated molecularly. The two most frequent species are *A. simplex sensu stricto* and *A. pegreffii*, with studies to date suggesting that the former is more pathogenic than the latter (Arai et al., 2014; Jeon and Kim, 2015; Quiazon et al., 2011; Rello Yubero, 2003; Romero et al., 2013; Suzuki et al., 2010). Molecular diagnosis has permitted the identification of human anisakiasis cases caused by both species (Arai et al., 2014; Arizono et al., 2012; Lim et al., 2015; Mattiucci et al., 2013; Umehara et al., 2007; Yera et al., 2016; and others), although use of this type of diagnosis is still uncommon.

Proteases participate in important biological processes in parasitic nematodes, being directly involved in their growth and survival, embryonic development, digestion of protein for nutrients, moulting and numerous metabolic processes (Britton and Murray, 2002; Hashmi et al., 2002; Pratt et al., 1992; Ray and McKerrow, 1992; Williamson et al., 2003b; Yu et al., 2014). They also play a vital part in host-parasite interaction such as invasion of the host, migration through host tissues, protection of the parasite against the host's immune

system and activation of inflammatory processes (Malagón et al., 2013; McKerrow et al., 2006). It would thus seem that the proteolytic activity of the parasites has a crucial role in their pathogenicity, as reported in the nematodes *Strongyloides stercoralis* (McKerrow et al., 1990), *Anisakis simplex* (Jeon et al., 2014; Sakanari and McKerrow, 1990), *Onchocerca volvulus* (Lustigman, 1993), *Trichinella spiralis* (Criado-Fornelio et al., 1992), and *Ancylostoma caninum* (Hawdon et al., 1995).

Despite their biological importance and their implications for health, the proteases of *Anisakis* have not been studied in depth. As has been suggested for other pathogenic agents, some proteases could act as targets for chemotherapy or vaccines, as is the case of the cathepsins, which are well conserved in nematodes (Britton and Murray, 2002; Sallé et al., 2018). At present, the treatment of anisakiasis is invasive and involves the extraction of the larvae by endoscopy in hospital, while there is still no effective pharmacological treatment. Consequently, further study of nematode biology is necessary if new, effective drug targets are to be found.

Proteases or peptidases can be divided into two large groups according to their mechanism of action: one, including the cysteine- and serine-proteases, which form covalent enzymatic complexes with the substrate, and the other, including aspartic and metallo-proteases, which do not form these covalent complexes (Rawlings et al., 2012; Rawlings and Barrett, 1993), with each group then having different inhibition strategies. These 4 groups of proteases are the most widely-studied in nematodes (Caffrey et al., 2013; Malagón et al., 2013; McKerrow et al., 2006; Sajid and McKerrow, 2002; Sakanari and McKerrow, 1990), although there are also other groups such as the glutamic or threonine proteases.

The aim of the present study was to perform a general characterization of proteolytic activity and to observe its variation during in vitro development of two sibling species of the

79 complex *Anisakis simplex s.l.: A. simplex s.s.* and *A. pegreffii*. Since both species can cause
80 anisakiasis, any differences observed may be due to their different pathogenicity.
81

82 2. Material and methods

83 *2.1. Sample collection and in vitro culture*

84 The L3 were extracted, as described previously (Molina-Fernández et al., 2018b), from the gut
85 cavity of blue whiting, *Micromesistius poutassou*, caught in the Cantabrian and
86 Mediterranean Sea and landed at Spanish ports. Immediately after extraction the larvae were
87 placed in cold 0.154 M NaCl solution in an ice bath to prevent their development. The
88 following larval stages were chosen for study: L3 recently extracted from the fish (L3-0h), L3
89 after 24 hours culture (L3-24h), L4, 24 hours after moulting (L4-24h), and L4 after 14 days
90 culture (L4-14d: 10 days after moulting to L4). In the case of L3-0h, the larvae were frozen
91 immediately after removal from the fish, having been first washed in cold sterile saline
92 solution. For the other stages, the larvae were axenized and individually placed in culture as
93 described previously (Iglesias et al. 1997, 2001). After attaining the stage desired, the larvae
94 were washed with the cold, sterile saline and frozen at -20 ºC until required. To obtain the
95 necessary larvae of each species of *Anisakis*, fish were chosen from different geographical
96 areas according to the predominant species of *Anisakis* in each. Fish landed at Ondarroa
97 (Cantabrian Sea, Northern Spain, zone FAO VIIIc) were used for larvae of *A. simplex s.s.* and
98 from Villajoyosa and Gandía (Mediterranean Sea, Eastern Spain, zone FAO 37.1.1) for *A.*
99 *pegreffii*. Molecular identification was carried out as described previously (Molina-Fernández
100 et al., 2018a, 2018b, 2015). Following DNA extraction from 28-30% of the collected larvae
101 from each of the fishing grounds, a polymerase chain reaction-restriction fragment length
102 polymorphism (PCR-RFLP) of the ribosomal DNA fragment ITS1-5.8S-ITS2 was performed

using the primers NC5 (forward) and NC2 (reverse) described by Zhu et al. (1998). RFLP was performed independently with two restriction enzymes, *TaqI* and *HinfI* Fast Digest (Thermo Scientific) at 65 °C and 37 °C for 10 min, respectively. The results were visualized by electrophoresis in 3% agarose gel, which permitted the sibling species of *A. simplex* complex to be identified according to the band pattern (D'Amelio et al., 2000; Martín-Sánchez et al., 2005a). Some larvae showed a mixed banding pattern between *A. simplex s.s.* and *A. pegreffii* with one or other restriction enzyme and were thus classified, for the purposes of this study, as hybrid type I larvae.

2.2. Preparation of Anisakis extracts

The larvae of each stage were homogeneized separately in a small volume of Tris/HCl 50 mM buffer with glycerine 20% w/v at pH 7.8, in order to stabilize the proteins and prevent their rapid degradation (Gianfreda and Scarfi, 1991; Iyer and Ananthanarayan, 2008), obtaining a final volume of 500 µl. The homogenates were then centrifuged at 19000 x *g* for 20 min, at 4 °C. The cell pellets were discarded and the supernatant fractions were used for the enzymatic activity assays. These fractions were diluted with the same buffer to a protein concentration of 2.5 mg/ml (Bradford, 1976). The larvae and the extract were kept in an ice bath for the entire process to prevent their degradation.

2.3. Proteolytic activity assays

Soluble extract of L3-0h of *A. simplex s.s*. was used to determine the effect of pH on proteolytic activity. A discontinuous system of buffers was employed, with pH increments of 1: citrate/HCl and citrate/NaOH from pH 2 to 7, Tris/HCl from 7 to 9 and glycine/NaOH from 9 to 11; always at a final concentration of 50 mM. The effects of dithiothreitol (DTT) (final concentration 0-10 mM) and $CaCl_2$ (final concentration 0-20 mM) on proteolytic activity were assayed. The use of DTT to reduce total proteolytic activity was ruled out, while the use of

127 $CaCl_2$ at 1 mM stabilized this activity. Changing ionic strength with NaCl did not improve
128 activity. Activity was determined by measuring the fluorescence emitted following the
129 degradation of the fluorogenic bodipy FL casein substrate (Thermo Fisher) with λ_{ex} 490 nm/λ_{em}
130 510 nm, using a fluorimeter (Fluostar Optima-BMG Labtech), where enzymatic activity was
131 monitored at 37 ºC for 60 min and measured every 1 min, in black microtiter plates. The final
132 concentrations in the reaction mixture were: 50 mM buffer, 50 µl extract/ml (125 µg
133 protein/ml), 1 mM $CaCl_2$ and 5 µg substrate/ml, for a final volume of 200 µl (Malagón et al.,
134 2011). The enzymatic reaction was started with the substrate. To standardize the procedure,
135 the area of greatest stability of the measurement was selected –corresponding to that
136 between 10 and 30 minutes – for calculation of proteolytic activity. Enzymatic activity was
137 expressed as a variation (Δ) of the fluorescence relative units (FRU) $min^{-1}mg^{-1}$ protein. After
138 determination of the pH values where peaks of maximum activity were observed, specific
139 buffers were used to cover pH±1 to improve determination of optimum pH. The buffer Tris-
140 maleic/HCl was used for pH 5.0, 5.5, 6.0, 6.5 and 7.0 and glycine/NaOH for pH 8.0, 8.5, 9.0,
141 9.5 and 10.0. After establishing the optimum pH values, the proteolytic activity of each of the
142 larval stages of *A. simplex s.s.* and *A. pegreffii* was determined.

143 *2.4. Inhibition assays*

144 To assess the contribution of the different protease classes to hydrolysis of the substrate,
145 protease inhibitors were assayed as previously described (Malagón et al., 2011). Inhibition
146 assays were carried out at the two pH values where maximum proteolytic activity had been
147 detected. The following control enzymes and specific inhibitors were used for each group of
148 proteases, with their final concentration in the reaction mixture shown: pepsin (64 U/ml) and
149 pepstatin A (0.02mM), respectively, for the aspartic proteases; thermolysin (0.2 U/ml) and
150 1,10-phenanthroline (2 mM), for the metalloproteases; papain (0,24 U/ml) and l-trans-

epoxysuccinyl-leucylamide-(4-guanidine)-butane (E64, 0.05 mM), for the cysteine proteases; chymotrypsin (0.1 U/ml) and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 2 mM), for the serine proteases. The quantity of each inhibitor was sufficient to totally inhibit the control enzyme from time 0. A solvent (0.5% methanol, used for pepstatin A and 1,10-phenanthroline) control was also performed. Inhibition assays were carried out at pH 6.0 and pH 8.5. The final concentrations in the reaction mixture were: buffer 50 mM, $CaCl_2$ 1 mM, enzymes and inhibitors as described above, 50 µl of extract/ml (125 µg protein/ml), 5 µg of substrate/ml. The addition of reducing agents (such as DTT or L-cysteine) to the reaction mixture did not significantly affect cysteine protease activity. The ideal incubation time for the stabilization of the interaction between the extract and the inhibitor was established as 5 minutes. The reaction was initiated by adding the substrate bodipy FL casein. Measurements were taken every minute for 60 minutes, at 37 ºC. The most stable part of the activity curve, between 10 and 30 minutes, was selected for data processing. The effect of inhibitors was expressed as percentage of inhibition (%I), determined as: %I = 100 – [(mean ΔFRU min^{-1} mg^{-1} protein in presence of the inhibitor/mean ΔFRU min^{-1} mg^{-1} protein in absence of the inhibitor) × 100].

2.5. Statistical study

The aim of the statistical study was to compare the proteolytic activity of *A. simplex s.s.* and *A. pegreffii* in each of the developmental stages studied and the evolution of this activity during the development of each species, while also analyzing the differences in the contribution of of each type of protease to this activity. This study was carried out using the program SPSS version 22.0 for Windows. Variance analysis (ANOVA) was also performed, after determining that the residuals of the variables followed a normal distribution using the Shapiro-Wilk test ($p>0.05$ in all cases) and with the aid of Q-Q plots. Following ANOVA, a *post*

hoc study was carried out using the Bonferroni test for the variables which had shown significant differences. The significance level was designated as $p<0.05$.

3. Results and discussion

3.1. Molecular identification

A total of 270 larvae from the port of Ondarroa (Cantabrian Sea) were used, of which 75 were analyzed by PCR-RFLP (27.8% of the larvae), 90.7% (68 larvae) being identified as *A. simplex s.s.*, 8.0% (6 larvae) as *A. pegreffii* and 1.3% (1 larva) as a type I hybrid of the two species. A total of 215 larvae from the Mediterranean Sea ports of Villajoyosa and Gandía were employed, of which 65 were analyzed (30.2% of the larvae), 90.8% (59 larvae) being identified as *A. pegreffii*, 1.5% (1 larva) as *A. simplex s.s.* and 7.7% (5 larvae) as type I hybrids of the two species. These data coincide with the known distribution of these species of *Anisakis*, with *A. pegreffii* more frequent in the western Mediterranean Sea and *A. simplex s.s.* in NE Atlantic Ocean, although with certain sympatric zones in the seas to the south and west of the Iberian Peninsula (Martín-Sánchez et al. 2005; Mattiucci and Nascetti 2008; Molina-Fernández et al. 2015, 2018b; Mattiucci et al. 2018).

3.2. Proteolytic activity

Activity of L3-0h de *Anisakis simplex s.s.* tended to be maximum at pH 6.0 and 8.5. It has also been observed that $CaCl_2$ (1 mM) favours proteolytic activity (Gianfreda and Scarfi, 1991; Iyer and Ananthanarayan, 2008; Kocher and Sood, 1998) and helps to maintain the stability of the proteases (Morris and Sakanari 1994). The use of reducing agents (such as DTT) in the reaction mixture may facilitate the action of the cysteine proteases, although, under the experimental conditions of the present study, it reduced total proteolytic activity. This may be due to the ability of the sulfhydryl groups of the reducing agents to form a complex with the metal ion

of some proteases, thus inhibiting their action (Coombs et al., 1962; Rufo et al., 1990) and affecting its measurement.

At pH 6 (Fig. 1), the proteolytic activity throughout the development of the two species was similar, although, the L4 showed significantly greater activity than L3 in the two species ($p<0.001$). It has been suggested that L3 of *Anisakis* do not ingest food orally until moulting to L4 (Sommerville and Davey, 1976; Yasuraoka et al., 1967). This change in the uptake of nutrients may require the action of different proteases for digestion in the intestine. Dziekońska-Rynko et al. (2003) reported greater activity of several digestive hydrolases in L4 *A. simplex* than in L3, relating it to changes in feeding mechanisms taking place after moulting to L4. These data suggest that the proteolytic activity detected at this pH may be related, in particular, to digestive processes involving the parasite's nutrition. The inhibition assays (Fig. 3) show that most of this activity during the development of *A. simplex s.s.* and *A. pegreffii* was due to aspartic (34-54%) and metallo-proteases (40-52%). The former are digestive proteases mainly associated with nutrition, in both parasites and free-living nematodes (Brown et al., 1995; Chang et al., 2011; Geldhof et al., 2000; Hawdon et al., 1989; Hwang et al., 2010; Williamson et al., 2003a, 2003b; Yang et al., 2009), and, according to Delcroix et al. (2006), aspartic and cysteine proteases assume the same role in invertebrate digestion as trypsins in that of vertebrates. Iglesias et al. (2001) showed that the development of L4 to adulthood in *A. simplex* is determined by the presence of pepsin (an aspartic protease), at least in culture. The pepsin aids availability of predigested peptides in the medium, facilitating their assimilation by the parasite. This can also occur in vivo through the pepsin from the glandular chamber of the stomach in cetaceans, the definitive hosts of *Anisakis* spp., as has also been suggested for the related nematode *Ascaris suum* (Rhoads and Fetterer, 1998; Rhoads et al., 1998). In *Hysterothylacium aduncum*, the addition of pepsin to the culture

222 medium was found to significantly reduce aspartic protease activity in the parasite, making
223 use of the exogenous contribution from either the culture medium or the digestive system of
224 the fish, the definitive hosts of this nematode. The greater expression of aspartic proteases
225 in the absence of pepsin suggests that these type of proteases have a digestive function in
226 these nematodes (Malagón et al., 2011). Furthermore, it has been observed that infective L3
227 of *A. simplex* stimulate pepsin expression in the stomach of guinea pigs, an experimental host
228 of this anisakid (Dziekońska-Rynko et al., 1997). At pH 8.5 total proteolytic activity was greater
229 in *A. pegreffii* than in *A. simplex s.s.* ($p<0.01$), although showing a similar trend in both species
230 (Fig. 2). Maximum activity was found in L4-24h in both species. However, the differences
231 between larval stages were only significant in *A. pegreffii* ($p<0.03$). No aspartic proteases were
232 detected at this pH since they are only active at acidic pH values.

233 While, at pH 6.0, the metalloproteases shared most of the proteolytic activity (40-52%)
234 with the aspartic proteases (Fig. 3), at pH 8.5 they accounted for the greater part of the
235 activity (40-75%; Fig. 4). In nematodes these proteases are involved in resisting the host's
236 immune system (Culley et al., 2000), in the moulting process (Hong et al., 1993; Rhoads et
237 al., 1997) and in nutrition (Rhoads and Fetterer, 1998). Metalloaminopeptidases have been
238 described in *Ascaris suum* (en L3, L4 and adults) and in the sheep stomach nematode
239 *Haemonchus contortus*, suggesting that they have an important role in digestion (Newton,
240 1995; Rhoads and Fetterer, 1998). However, other aminopeptidases detected in both *A.*
241 *simplex* and in the anisakid *Pseudoterranova decipiens* have not been implicated in digestion
242 but in the activation of biological molecules (proenzymes, prohormones) and in moulting
243 (Davey and Sommerville, 1974; Sakanari and McKerrow, 1990). Another Zn-protease has been
244 implicated in the eclosion and moulting of *H. contortus* (Gamble et al., 1989; Rogers, 1982).
245 This functional diversity might explain their high level of activity in all larval stages and at the

two pH values studied (Figs. 3 and 4). In *H. aduncum*, the metalloproteases also showed the greatest proteolytic activity (60-90%), at least at pH 5.5 (Malagón et al., 2011). It has been suggested that in the gastrointestinal tract of the final host the metalloproteases of the nematode parasite complete the digestion initiated by the pepsin and other enzymes from the host (Rhoads et al., 1998). However, in the L3-0h, collected from the host fish, the contribution of the metalloproteases to total activity was significantly higher ($p<0.03$) in *A. simplex s.s.* (70%) than in *A. pegreffii* (41%). These metalloproteases may contribute to the significantly greater capacity for penetration of the gut wall of laboratory animals by L3 of *A. simplex s.s.* (Romero et al., 2013).

As can be observed in the results (Figs. 3 and 4), the activity of cysteine protease in the species of *Anisakis* studied was low in almost all developmental stages at both pH values. It is noteworthy that, at pH 6.0, the L3-0h from the host fish showed greater activity in *A. simplex s.s.* (6-fold; $p=0.05$) than in *A. pegreffii*, while the opposite occurred (>2-fold in *A. pegreffii*) in the subsequent developmental stages (in the cetacean host). However, these data are difficult to interpret, particularly in view of their low activity (<12% of total proteolytic activity) and the great diversity of cysteine proteases reported from parasitic nematodes (Jasmer et al., 2001; Pratt et al., 1992; Rehman and Jasmer, 1999; Shompole and Jasmer, 2001) with a wide variety of, often vital, functions. These enzymes are present in both larvae and adults, suggesting involvement in nutrition, tissue penetration and defense against the host's immune system, in moulting, in development and maturation or in the adaptation of the nematode to the definitive host (Lustigman et al., 1996; Malagón et al., 2010b; McKerrow, 1995; M. L. Rhoads et al., 1998; Tort et al., 1999).

At pH 8.5 (Fig. 4), the activity of serine protease in *A. pegreffii* was <5% in all larval stages except L3-0h, in which it accounted for almost a third of total activity (32%), while,

during the development of *A. simplex s.s.*, activity remained between 14-19% of the total. The difference in activity between the two species is significant at all stages ($p<0.05$) except in L3-0h ($p=0.09$). At pH 6.0 (Fig. 3), activity was even lower in both parasites (<12%) while statistical comparison of the species showed that activity was greater in *A. simplex s.s.* ($p\leq0.05$), except in the most highly-developed stage (L4-14d; $p=0.20$). This type of enzyme is involved in the pathogenicity of the parasite and has a vital role in penetration of the host tissues. Sakanari and McKerrow (1990), in their examination of the products of excretion/secretion of L3 of *A. simplex*, found, in addition to metalloaminopeptidase, a trypsin-like serine protease (previously reported by Matthews, 1984), accounting for 80% of total secretory activity, which they implicated in the pathogenicity of the parasite, attributing to it the penetration of the larva in the host tissues. Morris and Sakanari (1994), working with L3 extracts, identified two serine proteases: one, trypsin-like, 89% similar to porcine trypsin and of the same Mw as that secreted, and, another, 85% similar to one secreted by the pathogenic bacteria *Dichelobacter nodosus*, which is able to degrade the elastin, keratin and collagen of cell tissues. Collagenolytic activity associated with serine proteases and metalloproteases has previously been described in *H. aduncum*, in stages L3, L4 and adults (Malagón et al., 2010a). It is thus clear that several workers have implicated the serine proteases, including those of L3 of *A. simplex*, in the invasion and penetration of host tissues. As reported previously, *A. pegreffii* is less pathogenic than *A. simplex*. This may also be due to the significantly lower serine protease activity of the former at pH 6.0, particularly in L3, the stage which must establish itself in the definitive host, at 37 ºC.

At pH 6.0, all proteolytic activity was inhibited by the joint activity of the inhibitors used (Fig. 3). However, at pH 8.5, a part of the activity (4-42%) was not inhibited by any of the inhibitors (Fig. 4). In similar studies on *H. aduncum* at pH 5.5, the uninhibited activity was

minimal (<5%), while, at pH 4.0 it was high (10-60%) (Malagón et al., 2011). This suggests that these nematodes may have special proteases which are not sensitive to general inhibitors or may have unusual types of protease, such as threonine or glutamic proteases. This study has found interspecific differences in some aspects related to pathogenesis in these and other nematodes and which could feasibly contribute to the differences in pathogenicity previously observed in these two species of *Anisakis*. Further research is required to clarify these questions.

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Compliance with ethical standards.

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Legends to figures:

Figure 1.- Proteolytic activity measured as optimal bodipy FL casein cleaving activity at pH 6.0 in *Anisakis simplex s.s.* and *A. pegreffii*, during its in vitro development. Each point is the mean of three to five experiments in triplicate.

Figure 2.- Proteolytic activity measured as optimal bodipy FL casein cleaving activity at pH 8.5 in *Anisakis simplex s.s.* and *A. pegreffii*, during its in vitro development. Each point is the mean of three to five experiments in triplicate.

Figure 3.- Inhibition percentage of optimal bodipy FL casein cleaving activity at pH 6.0 in *Anisakis simplex* complex, during its in vitro development. Each inhibition percentage is the mean of three to five experiments in triplicate. Top: *A. simplex s.s.*. Bottom: *A. pegreffii*.

Figure 4.- Inhibition percentage of optimal bodipy FL casein cleaving activity at pH 8.5 in *Anisakis simplex* complex, during its in vitro development. Each inhibition percentage is the mean of three to five experiments in triplicate. Top: *A. simplex s.s.*. Bottom: *A. pegreffii*.